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Application of the Fast-evaporation Sample Preparation Method for Improving Quantification of Angiotensin II by Matrix-assisted Laser Desorption/Ionization

Anthony J. Nicola,[1,3] Arkady I. Gusev,[1,3] Andrew Proctor,[1] Edwin K. Jackson[2] and David M. Hercules[3]*

[1] Department of Chemistry, University of Pittsburgh, Pittsburgh, PA 15260, USA
[2] Department of Clincial Pharmacology, University of Pittsburgh, Pittsburgh, PA 15260, USA
[3] Present address: Department of Chemistry, Vanderbilt University, Nashville, TN 37235, USA

The fast-evaporation method of sample preparation has been applied for quantitative analysis using matrix-assisted laser desorption/ionization (MALDI) mass spectrometry. An instrumental protocol focusing on improvement of shot-to-shot repeatability and compensation for signal degradation has been developed for quantification of angiotensin II using the fast-evaporation technique and an internal standard. The fast-evaporation method was compared to the standard method of sample preparation (using a multicomponent matrix) in the quantitative analysis of angiotensin II, and found to be superior in several respects. Improvement in sample homogeneity using the fast-evaporation method enhanced both point-to-point repeatibility and sample-to-sample reproducibility. The relative standard deviations of the analyte/internal standard ratios (point RSD) were decreased by a factor of three compared to those obtained using the multicomponent matrix method. The average point RSD was found to be *ca.* 5% for the fast-evaporation technique. Two internal standards were evaluated for quantification of angiotensin II. The better one, 1-SAR-8-Ile angiotensin II, yielded a relative standard deviation of the standard curve slope of *ca.* 2.2% over two orders of magnitude of concentration (45 nM to 3000 nM), an improvement by a factor of two over the standard preparation method. Renal microdialysate samples, spiked with angiotensin II and the internal standard 1-SAR-8-Ile angiotensin II, were also analyzed using the fast-evaporation technique. The detection limit was calculated to be in the high attomole range (675 amol). Furthermore, the accuracy for a single determination of angiotensin II concentration in these samples was found to be 13.9% with a relative error of 8.19%.

Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) has become a powerful tool for the quantification of biomolecules.[1–8] Several problems still exist in MALDI quantitative analysis. The major problems are poor point-to-point signal repeatability and sample-to-sample reproducibility, as well as shot-to-shot signal degradation. There are two major ways to compensate for these deviations: use of an internal standard, and optimization of sample preparation. Use of an internal standard can minimize deviations in an analysis, so that the system response (signal) approaches linearity with respect to analyte concentration. A sample preparation protocol that focuses on matrix selection as well as on crystal homogeneity can be useful for improving the absolute signal repeatability/reproducibility of both analyte and internal standard, which results in further improvement of measurement accuracy of the analyte.

Use of an internal standard is vital for quantitative analysis by MALDI. The best internal standards could be isotope-labelled derivatives of the analyte, but these are restricted to use with low molecular weight compounds due to poor MALDI-time-of-flight resolution. In quantitative analysis of some low molecular weight biopolymers using isotope-labelled internal standards, standard curve correlation coefficients of 0.967–0.98 were achieved over two orders of magnitude of analyte concentration.[5] Quantitative analyses of higher molecular weight peptides and proteins using internal standards which are also large peptides/proteins[3,8] (α-lactalbumin and cytochrome *c* using myoglobin as the internal standard) resulted in standard-curve correlation coefficients of 0.970 to 0.982 over one order of magnitude of analyte concentration.[3]

MALDI has been successfully implemented for quantification of the immunosuppressant drug cyclosporin A in both methanol and blood, with cyclosporin D as the internal standard.[2] The percent relative standard deviation of the standard curve slope (slope RSD) was found to be 1.36% (correlation coefficient = 0.997), and the relative standard deviations of the analyte/internal standard intensity ratios (point RSD) fell in the range of 3–10%, over two orders of magnitude of concentration. The reported accuracy was *ca.* 11%.

For quantitative analysis of insulin,[4] both point RSD and slope RSD were improved by a factor of two when an internal standard with chemical properties similar to those of the analyte was used, compared to one with chemical properties different from those of the analyte. For optimum results, the (analyte + internal standard)-to-matrix molar ratio should be held constant.[4] In addition, significant suppression of the internal standard peak was observed when the analyte concentration was much greater than that of the internal standard.

Selection of an appropriate internal standard only partially addresses the problem of systematic deviations in quantitation, particularly when the analyte and internal standard have significantly different concentrations

* Author for correspondence.

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and chemical properties. Chemical differences between the analyte and internal standard lead to competition for incorporation into the matrix crystal structure. Differences in incorporation into the matrix structure would result in different optimum laser energies required to produce maximum signal intensities for each peak. Additionally, the intensities of the analyte and internal standard peaks will change differentially with deviations in laser energy. Quantitation is difficult under such conditions. To overcome these obstacles, selection of a reliable sample preparation method also becomes a key factor in MALDI quantitative analyses.

A sample preparation and matrix selection method which focuses on crystal homogeneity is an important factor in maximizing absolute signal repeatability/reproducibility (in addition to internal standard selection). In the quantitative analyses of some simple sugars,[7] 2,5-dihydroxybenzoic acid (DHB) was found to give the most intense peaks, the best signal reproducibility, and lowest detection limits, of all matrices tested. Nitrocellulose has been suggested as a substrate for improving crystal homogeneity in MALDI analyses of biomolecules.[9] In the analyses of several peptides, the absolute variation in signal intensity was significantly reduced when nitrocellulose was used as the substrate (7–19%), compared to the standard preparation method (20–60%). Another method for improving signal repeatability involved using multicomponent matrices[10,11] for quantification of insulin.[4,12] Signal repeatability and precision of the standard curve slope were both improved by a factor of two (point RSD = 6–12%, slope RSD = 1.4%) when a DHB/fucose/5-methoxysalicylic acid multicomponent matrix was used, compared with using only DHB as the matrix.[4,12]

A sample preparation method using fast evaporation of a matrix as a substrate surface has recently been suggested.[13,14] The samples were prepared by applying the matrix (α-cyano-4-hydroxycinnamic acid in a 1–2% water in acetone solution) to the probe surface. The analyte was deposited on top of the matrix crystal layer. The detection limit was found to be in the high attomole range (for Substance P), and a mass resolution of *ca.* 5700 (for a synthetic polypeptide of mass 2061) was achieved.[13] Some improvement in signal repeatability was also observed using a similar forced-evaporation sample preparation method.[15]

The goal of the present work is improvement of MALDI quantitative analysis using the fast-evaporation technique of sample preparation, and application of this method for quantification of angiotensin II. Angiotensin II, the major vasoconstrictive compound of the renin–angiotensin system, is responsible (to some extent) for increased overall blood pressure.[16] There is a great deal of interest in monitoring changes in the physiological levels of angiotensin II, and the effects of these changes on patients suffering from hypertension. These physiological levels can range up to 30 nM for angiotensin II in fluid drawn directly from the kidney.[17] It should be noted that renal microdialysis procedures yield *ca.* 30% recovery of the material from which the fluid is taken[18] (i.e., the concentration of angiotensin II in microdialysate fluid would be approximately 9 nM).

An analytical protocol for quantification will be developed, including selection of an appropriate internal standard. These results of this method of sample preparation will be compared to those obtained using a multicomponent matrix for shot-to-shot repeatability (signal degradation), point-to-point repeatability, and sample-to-sample reproducibility. The precision of the analyses (standard curve and estimated determination of analyte concentration) will be discussed, and the accuracy of angiotensin II analysis from renal microdialysate fluid, will be evaluated.

EXPERIMENTAL

Instrumentation

The instrument used in these experiments was a modified LAMMA 1000 (Leybold-Heraeus GmbH, Germany) time-of-flight mass spectrometer with a nitrogen laser (VSL337 ND, Laser Science Inc., Cambridge, MA, USA). The sample chamber was evacuated to a pressure of 2×10^{-6} mbar, while the analyzing chamber (flight tube and detection system) was held at a pressure of $2–4 \times 10^{-8}$ mbar. The positive ions were accelerated to 15 kV in front of the detector. The data-acquisition and secondary-information-processing software was written in-house 'GOOGLY', described elsewhere[2,4,12]). Normal processing of mass spectra includes linear background subtraction to improve the accuracy of peak-intensity measurements. The standard curve data are fitted using the method of linear least-squares.[19]

For investigations with the multicomponent matrix, the size of the laser beam at the point of interaction was *ca.* 100×150 μm, and the laser fluence was set to *ca.* 40–50% above the ionization threshold. For the fast-evaporation sample preparation technique, the laser beam was defocused to *ca.* 150×220 μm, and the laser fluence was set to *ca.* 25% above the ionization threshold. Use of a much smaller spot size (i.e., less 25×40 μm) led to a decrease in signal intensity in both investigations.

Standard curve sample preparation

The analyte used in these investigations was angiotensin II (FW 1046.2). Two internal standards were used: 1-SAR-8-Ile angiotensin II (FW 968.2) and 5-Val angiotensin II (FW 1034.2), both synthetic derivatives of angiotensin II. These were dissolved in high-performance liquid chromatographic (HPLC)-grade methanol to make stock solutions. Analyte and internal standards were obtained from Sigma Chemical Co. (St. Louis, MO, USA). HPLC-grade methanol and water were purchased from Fisher Scientific Co. (Pittsburgh, PA, USA).

Renal microdialysate samples

Angiotensin-free renal microdialysate fluid from rats was obtained using the protocol outlined elsewhere.[20] Each microdialysate sample was spiked with angiotensin II and the internal standard 1-SAR-8-Ile angiotensin II. The volume of each renal microdialysate sample was *ca.* 300 μL. No extraction was carried out, since the fluid is relatively clean, due to the filtration of the dialysis membranes. These samples were then transferred to plastic conical-bottom centrifuge tubes, evaporated to dryness using a Savant SpeedVac System

(Model SC110; Savant Instruments, Inc., Farmingdale, NY, USA), and reconstituted in 30 μL of a 1:1 water/methanol solution. All microdialysate samples were prepared using the fast-evaporation method of sample preparation (see later section), and subsequently washed using the procedure which was outlined elsewhere.[13] Briefly, a washing solvent was applied on top of the sample spot, and blown off by a stream of nitrogen.

Multi-component matrix

2,5-Dihydroxybenzoic acid (DHB, 20 mg/mL) and 5-methoxysalicylic acid (MSA, 0.6 mg/mL) were used to form a multicomponent matrix. DHB was purchased from Sigma Chemical Co., and MSA from Aldrich Chemical Co. Milwaukee, WI, USA). The matrix was prepared in a solution of 9:1 water/ethanol. The analyte was prepared by dissolving the solid in methanol at the highest concentration possible (in the range of 300–700 μM), and standards were prepared by serial dilutions of that stock solution. The standard curve concentrations ranged from 90 nM to 6000 nM. The internal standard (1-SAR-8-Ile angiotensin II) was prepared at a concentration of 660 nM. The analyte was mixed in a 1:1 ratio with the internal standard (IS), and this analyte/IS solution was mixed in a 1:1 ratio with the multicomponent matrix solution. This (analyte + IS)/matrix solution was then deposited onto a polished stainless steel substrate (spectrometer probe) in volumes of *ca.* 2 μL per sample.

Fast-evaporation sample preparation

The matrix used for this preparation was α-cyano-4-hydroxycinnamic acid (α-CHCA), purchased from Sigma Chemical Co. Several variations to the preparation method reported elsewhere[13] were used in the present work. A solution of α-CHCA was prepared in acetone at a concentration of 10 mg/mL (without water or trifluoroacetic acid (TFA)[13]). The volume, in μL, of this matrix solution which was deposited on the surface was approximately 1/10 the substrate area in mm^2. The areas of the substrates ranged from 25–100 mm^2; thus, the matrix/acetone solution was deposited onto the substrates in volumes of *ca.* 2.5–10 μL. This resulted in greater crystal thickness compared to previously published results, which we consider essential for improvement in signal reproducibility (as previously reported,[13] much less matrix was used for the matrix crystal layer, resulting in a much thinner layer).

A second solution of α-CHCA was prepared at a concentration of 10 mg/mL in 1:1 water + methanol (a saturated solution). The analyte internal standard (IS) standard curve solutions were mixed in a 1:1 ratio with the matrix. An equal part of water (instead of aqueous acid) was added to the analyte/IS mixture (already in pure methanol) to match the solvent of the matrix, so that the matrix crystal layer would not re-dissolve to any significant extent. The concentration range of the standard solutions was 45 nM to 3000 nM. The concentration of 1-SAR-8-Ile angiotensin II was 330 nM, while the concentration of the other internal standard (5-Val-angiotensin II) was 320 nM; *ca* 1.0 μL of (analyte + IS)/matrix solution was deposited on top of the matrix layer.

RESULTS AND DISCUSSION

Instrumental protocol for quantitative analysis

An instrumental protocol for MALDI quantitative analysis should result in improvement of point-to-point repeatability and compensation for shot-to-shot signal degradation[12] by optimizing the number of laser shots collected at each point on a given sample and the frequency of moving the spectrometer probe. This is a general approach for minimizing some deviations in quantitative analysis; a specific protocol should be developed for any matrix and sample preparation which is going to be used for quantitative analysis. To develop the instrumental protocol for quantification of angiotensin II using the fast-evaporation method, a solution of angiotensin II (358.1 nM) with the internal standard 1-SAR-8-Ile angiotensin II (330 nM) was deposited onto five separate stainless steel substrates using the fast-evaporation sample preparation method.

Sixty laser shots could be obtained from a single spot on the sample before the signal completely disappeared using the modified fast-evaporation method. We collected six consecutive mass spectra, consisting of ten laser shots each, from a single spot on the sample. Hence these spectra show the signal variation with sample usage at a single spot. This procedure was carried out for eight random spots on the sample, and for all five separate samples. It should be noted that only 20 laser shots per spot were collected using the original fast-evaporation technique.[13]

To improve the signal-to-noise ratio and point-to-point repeatability, the spectra from the first ten laser shots were compiled from each point on a given sample. The ratios of the analyte/internal standard peak intensities were then calculated for each of the six resultant composite spectra, along with the relative standard deviation for the data set. The same procedure was then carried out for the composite spectra of the second ten laser shots, the third ten, and so on. The average results (from all five samples) of the percent relative standard deviation (RSD) of analyte/internal standard ratios *vs.* number of laser shots, as well as absolute analyte signal intensity *vs.* number of laser shots, are shown in Fig. 1. The absolute intensity was normalized to 1, whereas the relative standard deviation is reported in actual units (percent).

The maximum signal intensity was obtained for the first forty laser shots, and the lowest relative standard deviation was achieved for the forty laser shots after the first ten. A higher percent RSD for the first ten shots can be explained by the presence of contamination at the surface, which causes deviations in the analyte/internal standard ratio (a similar effect was found in investigations using a multicomponent matrix[12]). To avoid such deviations in the analyte/internal standard ratio, the first 10 laser shots should be discarded. The increase in the percent RSD after 50 laser shots is due to the decrease in the signal-to-noise ratio (S/N) of the internal standard. This indicates that the deviations in the analyte-to-internal standard intensity ratios increase when the sample is consumed. *Thus, the optimum protocol is summation of the forty laser shots after the first ten have been discarded.*

To examine point-to-point repeatability, spectra from one spot were summed into a composite spectrum using only the 'optimum' spectra (the forty laser shots

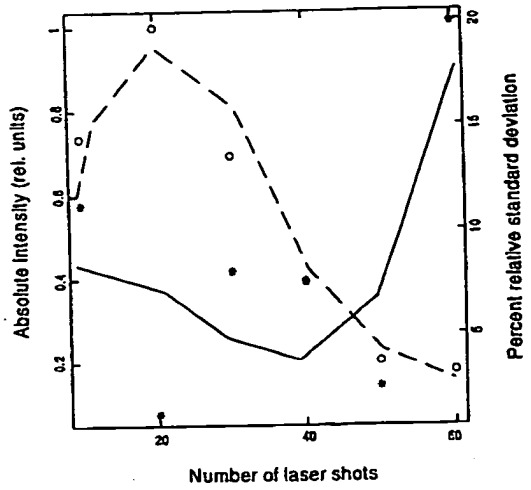

Figure 1. Measure of signal degradation due to ablation of the sample. Absolute signal intensity is normalized to 1.0. The concentration of angiotensin II was 358.1 nM, and the concentration of 1-SAR-8-Ile angiotensin II (internal standard) was 330 nM. Curves are fitted using a third-order polynomial approximation. Absolute signal intensity: o——; point RSD: *——.

collected after the first ten had been discarded). This was done for each of the eight spots on a given sample, and for all five samples. The point-to-point percent relative standard deviations (point RSD) ranged from 3% to 9%, with an average value of 7%. It should be noted that this is a result of composite spectra consisting of only 40 laser shots. Summation of more spectra will result in lower point RSD.

To compare sample-to-sample reproducibility *vs.* point-to-point repeatability, sixty laser shots were obtained from eight points on a given sample (a total of 480 spectra). These mass spectra were compiled into one composite spectrum. The same procedure was then carried out for the other four samples as well. The percent RSD of the analyte/IS intensity ratios (sample-to-sample) was *ca.* 5%. Using only the forty laser shots after the first ten ('optimum'), spectra consisting of 320 summed laser shots were compiled, resulting in a sample-to-sample percent RSD of *ca.* 3%. Thus, discarding the first and last ten laser shots (using only the 'optimum' forty laser shots) resulted in a decrease in a point-to-point and sample-to-sample RSD. Judging by this result, it is also clear that signal degradation is accompanied by distortion of the analyte/internal standard ratios, and hence an increase in deviation.

Comparison of fast-evaporation and multicomponent matrix techniques

Standard curve samples were analyzed according to the protocol outlined above for the fast-evaporation sample preparation technique. On each sample, five replicate mass spectra, each consisting of 300 summed laser shots, were collected from random points on sample. Thus, each spectrum required the use of eight points on a given sample, so that a total of 40 random spots were used on each sample; less than 20% of the total sample was consumed. For the multicomponent matrix investigation, we used the data collection protocol outlined elsewhere.[12] Briefly, the first fifteen laser shots were discarded, and the next 50 laser shots were collected.

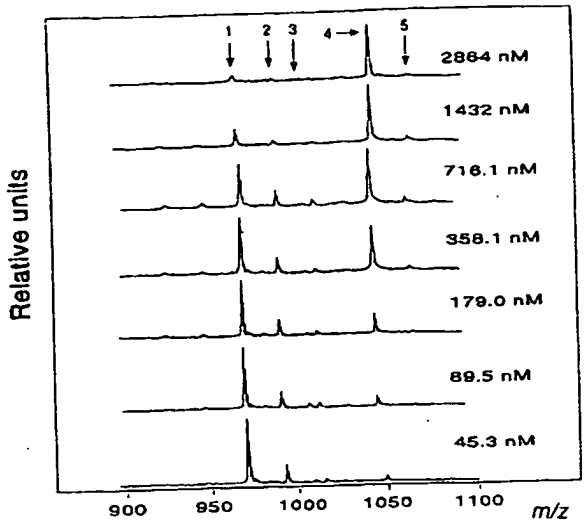

Figure 2. Mass spectra of angiotensin II (AII, 1047.2 Da) with the internal standard 1-SAR-8-Ile angiotensin II (1S8IAII, 969.2 Da) for the concentration range of 45 nM to 3000 nM, using the fast-evaporation sample preparation technique. The concentration of the internal standard was 330 nM. Each mass spectrum was normalized to 1 based on the highest peak. The following peaks are noted: 1: $[1S8IAII+H]^+$; 2: $[1S8IAII+Na]^+$; 3: $[1S8IAII+K]^+$; 4: $[AII+H]^+$; 5: $[AII+Na]^+$.

Again, five replicate spectra were collected for each sample, each consisting of 300 summed laser shots. Thus, six random spots were used for collection of each spectrum, and a total of 30 spots were used on each sample; less than 10% of each sample was consumed during the analysis. It should be noted that the crystal structure observed in the multicomponent matrix investigation was visually more inhomogeneous compared to that obtained with the fast-evaporation sample preparation method.

Figure 2 presents mass spectra of angiotensin II with the internal standard 1-SAR-8-Ile angiotensin II, obtained using the fast-evaporation technique. Each spectrum was normalized to 1, based on the highest peak. The major peaks for both the multicomponent matrix (not shown) and fast-evaporation techniques are due to the protonated molecules; cationization by sodium and potassium is small for both the analyte and internal standard. No overlapping with matrix peaks occurred for either compound. Enlarged spectra showing each isotopic peak cluster, obtained using the multicomponent matrix and fast-evaporation methods, are shown in Fig. 3(a) and (b) respectively. The mass resolution of the analyte isotopic peaks achieved for the fast evaporation technique gave $m/\Delta m$ *ca.* 2000 (full width at half maximum (FWHM)), and $m/\Delta m$ *ca.* 1150 (FWHM) for the multicomponent matrix investigation. This improvement in resolution using the fast-evaporation technique is consistent with previously published observations.[13]

Figure 4(a) and (b) show the standard curves obtained from the multicomponent matrix investigation and the fast-evaporation sample preparation technique, respectively. The equation of the line for the standard curve obtained in the multicomponent matrix investigation was

$$y = (1.43 \times 10^{-3})x + (5.86 \times 10^{-2}) \quad (1)$$

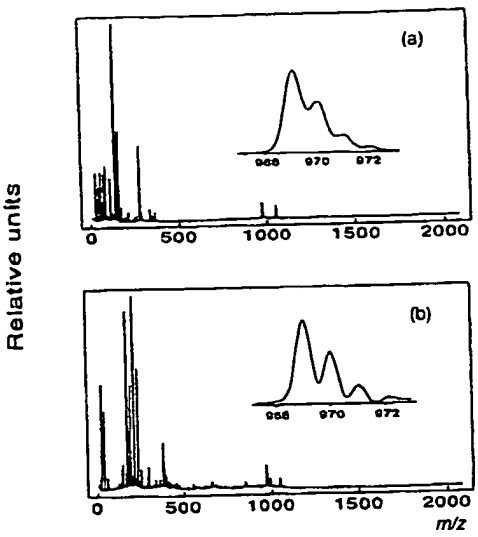

Figure 3. Mass spectra illustrating the resolution achieved with different sample preparation techniques. (a) Mass spectrum of angiotensin II (358.1 nM) with the internal standard 1-SAR-8-Ile angiotensin II (660 nM) using a multicomponent matrix. Resolution $(m/\Delta m) = 1150$. (b) Mass spectrum of angiotensin II (179 nM) with the internal standard 1-SAR-8-Ile angiotensin II (330 nM) using the fast-evaporation technique. Resolution $(m/\Delta m) = 2000$.

and for the fast-evaporation sample technique,

$$y = (2.12 \times 10^{-3})x + (4.06 \times 10^{-2}) \quad (2)$$

where, y is the analyte/internal standard intensity ratio, and x is the concentration in nM. The comparative results of the fast-evaporation and multicomponent matrix techniques are summarized in Table 1 for both identical concentration ranges and the whole concentration range for each method (results in parentheses). The detection limit in both cases was determined experimentally using the S/N ratio obtained for the smallest concentration of analyte (45 nM) and calculating the analyte concentration corresponding to S/N = 3. A theoretical detection limit of 11 mM, or 11 fmol of material per sample, was calculated for the multicomponent matrix method; the detection limit calculated for the fast-evaporation technique was 5.5 nM, or 2.8 fmol of material.

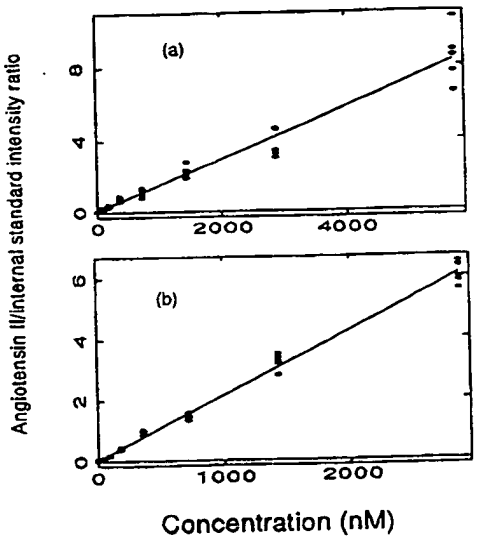

Figure 4. Standard curves obtained using multicomponent matrix and fast evaporation techniques. (a) Standard curve of angiotensin II using the multicomponent matrix technique. The concentration range is from 90 nM to 6000 nM. The internal standard was 1-SAR-8-Ile angiotensin II, at a concentration of 660 nM. (b) Standard curve of angiotensin II using the fast evaporation sample preparation technique. The concentration range is from 45 nM to 3000 nM. The internal standard was 1-SAR-8-Ile angiotensin II, at a concentration of 330 nM.

It is clear from these results that the fast-evaporation sample preparation technique yields superior results for quantitative analysis of angiotensin II. The fast-evaporation technique gave a lower slope RSD that did the multicomponent matrix method. In addition, the average point RSD for the fast-evaporation technique was lower by a factor of three compared to that for the multicomponent matrix method, showing an improvement in point-to-point repeatability. An F-test[19] was carried out for this comparison of sample preparation methods, confirming that the variance from the multicomponent matrix technique is significantly greater than that in the fast-evaporation technique at the 95% confidence limit.

Quantification of angiotenin II using both sample preparation methods can be compared to using a DHB/MSA/fucose multicomponent matrix for quantification of cyclosporin A[2], over a dynamic range similar to that used for angiotensin II (see Table 1). It is clear that using a multicomponent matrix for cyclosporin A analysis yielded superior results over those using the same sample preparation method for quantification of angiotensin II. It is interesting to note, however, that quantitative analysis of angiotensin II using the fast-evaporation technique gave results comparable to those obtained for cyclosporin A using a multicomponent matrix. This suggests that the multicomponent matrix method of sample preparation may be more suitable for compounds which tend to be cationized either by sodium or potassium (such as cyclosporin A), whereas the fast evaporation technique might be more appropriate for compounds which tend to ionize as the protonated-molecule species. Preliminary experimental results of analysis of cyclosporin A using the modified fast-evaporation sample preparation method in our laboratory seem to validate this hypothesis.

Comparison of internal standards

The possible use of more than one internal standard is relevant, because other species present in biological fluids may interfere with the internal standard. It would be advantageous to have more than one internal standard that yields reproducibile results in quantitative analyses of angiotensin II in biological fluids. In order to select the best internal standard for future investigations, two internal standards were compared: 1-SAR-8-Ile angiotensin II (FW 968.2, conc. 330 nM), and 5-Val angiotensin II (FW 1034.2, conc. 320 nM). The linear dynamic range for analyte concentrations was the same for both internal standards. The equation of the line for the standard curve using 1-SAR-8-Ile angiotensin II was shown as eqn (1). The equation of the line for the standard curve using the internal standard 5-Val angiotensin II was

$$y = (2.03 \times 10^{-3})x + (8.42 \times 10^{-2}) \quad (3)$$

Comparison of results for the two internal standards

Table 1. Comparison of results of quantitative analyses using fast-evaporation and multicomponent sample preparation techniques. Number in parentheses are for extended quantification range

Technique	Point RSD (%)	Slope RSD (%)	Correlation coefficient	Detection Limit nM	Detection Limit fmol	Precision[d]	Quantitative Range (nM)
Fast evaporation[a] angiotensin II	2–7% (2–7%)	2.6% (2.2%)	0.992 (0.996)	5.5	2.8	7% at 1400 nM[e]	90–3000 (45–3000)
Fast evap.[b] angiotensin II	5–9% (5–8%)	2.7% (2.4%)	0.991 (0.994)	5.5	2.8	8% at 1400 nM[e]	90–3000 (45–3000)
Multicomponent matrices angiotensin II	10–16% (10–20%)	5.5% (4.5%)	0.966 (0.976)	11	11	13% at 1400 nM[e]	90–3000 (90–6000)
Multicomponent matrices cyclosporin A[c]	3–10%	2.1%	0.996	8.3 nM		11% at 1200 nM[e]	90–2500

[a] Internal Standard = 1-SAR-8-Ile angiotensin II.
[b] Internal Standard = 5-Val angiotensin II.
[c] Calculated from data reported elsewhere (Ref. 2).
[d] CV% at 95% confidence level.
[e] Concentration for which precision was calculated.

are also presented in Table 1. It was found that use of 1-SAR-8-Ile produced slightly better results than those obtained using 5-Val angiotensin II as the internal standard. Here, the slope RSD was *ca.* 2.2%, the standard curve correlation coefficient was 0.996, and the average point RSD was *ca.* 5%. The results indicate that either internal standard would be suitable for quantitative analyses of angiotensin II. An F-test (95% confidence level) confirms that the variance in quantification of angiotensin II using the internal standard 5-Val angiotensin II is not significantly greater than that obtained with the internal standard 1-SAR-8-Ile angiotensin II.

Intensity measurements

All of the data reported above used linear background subtraction, and measurement of all peaks in the isotopic cluster (a mass range of 4 u). However, incorrect baseline subtraction (a result of non-linear baseline appearance in MALDI spectra) could introduce a great deal of error into quantitative measurements. Linear background subtraction was used previously[2,4,12] for peak intensity measurements, and found to give consistent results. However, it is important to evaluate several different methods of peak intensity measurement.

One such method is a procedure described by Liu and Koenig for automatically estimating a background profile.[21] The authors assume that the background can be adequately described by a parabola. A first estimate of the background is determined by fitting the chosen curve to all data points. By rejecting points which lie well outside this curve (outliers) a new set of background points can be determined. New background profiles and hence an improved set of background points are generated iteratively until further iteration results in little or no change in the background profile.[21] In the present work, the background function chosen was the cubic spline described by Reinsch.[22,23] The choice of the spline allows a much more flexible background profile than can be obtained by simply using a parabola or even higher order polynomials. Once variable parameters, like the outlier rejection criterion and the spline flexibility, have been chosen, the process is completely automatic. This ultimately removes much of the subjectiveness from the background profile determination.

The methods of baseline subtraction and intensity measurements which were evaluated were: (i) peak area (total counts) using all of the isotopic peaks in the cluster (mass range of 4 u), using both linear and spline background subtraction; (ii) peak area (total counts) using the FWHM of the $[M+H]^+$ peak after spline baseline subtraction; (iii) the ratio of the peak area (total counts) using the FWHM of the $[M+H]^+$ peak divided by the FWHM for the peak, using the spline baseline (to compensate for differences in peak width and shape); and (iv) peak height using the spline baseline. Baseline subtraction was performed by 'GOOGLY' software. Comparison of these methods was carried out for the standard curve spectra of angiotensin II obtained using the fast-evaporation technique and the internal standard 1-SAR-8-Ile angiotensin II. For all of the methods, the analyte-to-internal standard intensity ratios were calculated, and the comparative results for point and slope RSD are shown in Table 2. An F-test (95% confidence limit) between the method of linear background subtraction over 4 u (lowest slope RSD) and the method of spline background subtraction over 4 u (highest slope RSD) showed that the variance in the spline background subtraction investigation was not significantly greater than that associated with linear background subtraction. Thus, none of the methods examined showed a significant advantage over the others in this mass range. However, using spline baseline subtraction for peak intensity measurements could be very useful for analyses of higher molecular weight compounds, where factors such as increased metastable decay can cause incorrect background subtraction.

Measurement precision

The precision of the measurement of an unknown concentration using an intensity ratio from the standard curve can be estimated by using the following formula:[19]

$$s_{x_0} = \frac{s_{y/x}}{b}\left(\frac{1}{m} + \frac{1}{n} + \frac{(y_0 - \bar{y})^2}{b^2 \Sigma_i (x_i - \bar{x})^2}\right)^{1/2} \quad (4)$$

where y_0 is the experimental value of y from which the concentration value x_0 is to be determined, s_{x0} is the estimated standard deviation of x_0, $s_{y/x}$ is the standard deviation of y-residuals, b is the slope of the standard curve line, m is the number of replicates taken at the given y_0, and n is the number of points on the regression line. The confidence interval of the mean (at the 95% confidence limit) was calculated using the following formula:

$$\mathrm{CIM}_{95\%} = \bar{x} \pm s_{x0}/n^{1/2} \quad (5)$$

where $\bar{x}$ is the experimental mean, t is the t-statistic at the 95% confidence level, s_{x0} is the standard deviation, and n is the number of replicates measured.

Based on Eqns 4 and 5, the estimated single-measurement precision of the fast-evaporation technique and the multicomponent matrix method were compared for quantification of angiotensin II, and the results are reported in Table 1. For the multicomponent matrix method the estimated precision (at a concentration of 1400 mM) was found to be *ca.* 13%. For the fast-evaporation technique using the internal standard 1-SAR-8-Ile angiotensin II, the estimated precision (at a concentration of 1400 nM) was found to be *ca.* 7%, whereas for the use of the internal standard 5-Val angiotensin II, the estimated precision (at a concentration of 1400 mM) was found to be *ca.* 8%.

Renal microdialysate samples

Five microdialysate samples were spiked with angiotensin II and 1-SAR-8-Ile angiotensin II to give concentrations of 8.95 nM and 11.50 nM, respectively. Since the fluid is relatively clean, no extraction procedures were carried out. It should also be noted that preparation of these spiked samples involved concentration of each by a factor of ten (see Experimental section). The samples were then prepared by the same fast-evaporation technique as that used for the standard curve samples, with the addition of washing of the samples. Several solvents were examined for this washing procedure: water, 0.1% TFA in water, 1:1 methanol + water. Washing times ranged from 1–4 min. The best solvent for washing of samples was found to be 0.1% TFA in water, and the optimum amount of time for washing was 3 min. Longer washing times did not yield any significant improvement. Figure 5(a) and (b) present mass spectra of microdialysate samples without washing and with washing (0.1% TFA), respectively. It is evident that washing of the samples resulted in a reduction in the peaks corresponding to sodium and potassium cationization of the analyte and internal standard, as well as in the peaks from Na^+ and K^+. Furthermore, the molecular-ion intensities for both the analyte and internal standard were increased by a factor of *ca.* 2. It is also clear from Fig. 5(a) and (b) that there were no interfering peaks from possible organic contaminants in the microdialysate fluid.

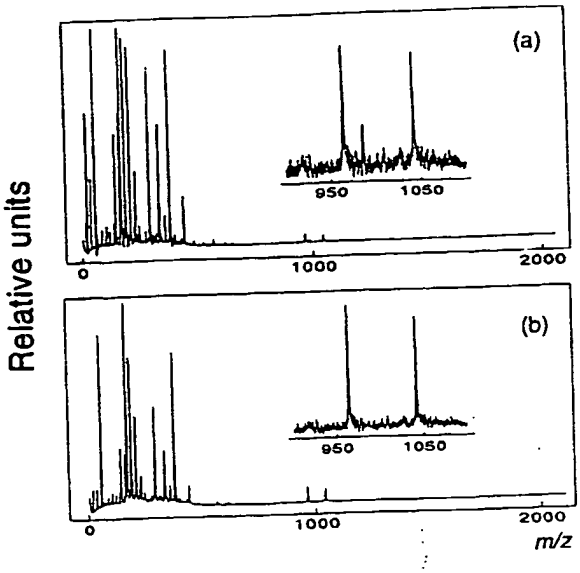

Figure 5. Mass spectra illustrating the effects of washing, on renal microdialysate samples. The concentration of angiotensin II was 8.95 nM, and the concentration of 1-SAR-8-Ile angiotensin II was 11.50 nM. (a) Mass spectrum of spiked microdialysate sample without washing. (b) Mass spectrum of spiked microdialysate sample washed with 0.1% TFA for three minutes.

Five replicate spectra, each consisting of 300 summed laser shots, were obtained from each microdialysate sample. These spiked microdialysate samples were analyzed to determine the accuracy (relative error, (RE%) the absolute error relative to true quantity) and precision (CV%) of angiotensin II concentration measurements using the fast-evaporation technique. In addition to these samples, two standards were analyzed to obtain the standard curve correction. The relative error was calculated to be 8.19% with a CV% of 7.33%. The accuracy for determination of angiotensin II concentration from a single spiked sample was calculated using the confidence interval of the mean (Eqn (5)). The $\mathrm{CIM}_{95\%}$ was calculated to be 8.24 ± 0.53 nM, corresponding to a CV% of 6.5%. This yields a single determination accuracy of 13.9%. The detection limit was found to be 1.345 nM, or 675 amol angiotensin II per sample. This indicates that MALDI quantitative analysis using the fast-evaporation technique is capable

Table 2. Comparison of intensity measurement methods

Method	Average point RSD (%)	Slope RSD (%)
Linear background subtraction (Range = 4 u)	5	2.2
Spline background subtraction (Range = 4 u)	8	2.8
Spline background subtraction using FWHM of $[M+H]^+$ peak	9	2.4
Spline background subtraction using FWHM of $[M+H]^+$ peak normalized to 1 u width	7	2.7
Spline background subtraction using peak height above baseline	8	2.5

of reaching physiological levels of angiotensin II in renal microdialysate fluid.

CONCLUSIONS

An experimental protocol for implementation of the fast-evaporation sample preparation technique in MALDI quantitative analyses of angiotensin II has been developed. The results clearly indicate that this method of sample preparation is superior to the standard sample preparation method (using a multi-component matrix). The fast-evaporation technique yielded improved point-to-point repeatability as well as improved sample-to-sample reproducibility over the multicomponent matrix method, which is a reflection of better crystal homogeneity. In addition, the precision of the standard curve was improved by a factor of two; as a result, the estimated precision of a single measurement (found to be *ca.* 7% at a concentration of 1400 nM), was also enhanced by a factor of two. Two internal standards (1-SAR-8-Ile angiotensin II and 5-Val angiotensin II) were proven useful for quantitative analysis of angiotensin II. A comparison of the two showed that there is no significant advantage (increased precision or repeatability) to using one over the other. Furthermore, it was found that spline background subtraction did not yield significantly better results than linear background subtraction for peak intensity measurements.

Finally, the determination of angiotensin II concentrations from spiked microdialysate samples gave a relative error of 8.19%, a single determination accuracy of 13.9%, and a theoretical detection limit of 675 amol.

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